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TCDD Alters Pituitary-Adrenal Function I: Adrenal Responsiveness to Exogenous ACTH

LORELLE L. BESTERVELT,*† YONG CAI,* DOUGLAS W. PIPER,* COLLEEN J. NOLAN,*†
JEFF A. PITT*† AND WALTER N. PIPER*‡[1]

**Toxicology Program, School of Public Health, †Reproductive Sciences Program, and ‡Department of Pharmacology, Medical School, The University of Michigan, Ann Arbor, MI 48109-2029*

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BESTERVELT, L. L., Y. CAI, D. W. PIPER, C. J. NOLAN, J. A. PITT AND W. N. PIPER. *TCDD alters pituitary-adrenal function I: Adrenal responsiveness to exogenous ACTH.* NEUROTOXICOL TERATOL 15(6) 365-370, 1993.—Plasma ACTH concentrations in 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated rats (50 μg/kg; single, oral dose) were 2.1-, 2.1-, 2.9-, 1.7-, 1.5-, 2.0- and 3.0-fold greater than control values, respectively, at days 1, 3, 5, 7, 10, and 14. At days 1 and 5 plasma corticosterone concentrations were increased 5.1- and 8.0-fold, respectively; whereas, at days 10 and 14 they were depressed to values of 50% and 39% of controls, respectively. Adrenal glands were exised from rats treated with TCDD and corticosterone production was assessed. Basal corticosterone concentrations produced by treated adrenals were depressed to 81%, 72%, and 71% of control values at days 5, 7, and 14, respectively. Corticosterone secretion by ACTH stimulated adrenals was equivalent to controls. These findings suggest that TCDD exposure decreases the bioactivity of the ACTH secreted by the anterior pituitary.

2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin Adrenocoticotropin Pituitary gland Adrenal gland Corticosterone

HALOGENATED aromatic hydrocarbons (HAHs) represent a chemical family of highly toxic environmental contaminants that include the polychlorinated and polybrominated biphenyls, the polychlorinated dibenzofurans, and the polychlorinated dibenzo-*p*-dioxins. The prototypical HAH is 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD has been studied more extensively than any other HAH because it is considered to be the most toxic and biologically potent congener (24,26). TCDD is lipophilic, stable and resistant to chemical and biological degradation. As a consequence of these physiochemical properties it has a propensity to persist in the environment and to bioaccumulate in the food chain, posing a potential risk to human health (16). TCDD is a contaminant formed as a by-product in the manufacture of products from chlorinated compounds (22,27), during the combustion of chlorinated materials (5,7,13), and in the process of bleaching paper pulp (2,30).

TCDD induced hirsutism, alopecia, and chloracne reported in various mammalian species suggest that adverse effects of TCDD may be mediated through alterations in endocrine function (1,17,18). Anorexia, progressive weight loss, and hypoglycemia have been reported both in humans and animals after TCDD exposure (9,11,15,16). These adverse effects are symptoms of adrenocortical insufficiency (Addison's disease), suggesting that TCDD exposure may compromise adrenocortical function. This suggestion is supported by the observations that [^{14}C]TCDD accumulates in the adrenal gland (8, 23,25) and that pathologic lesions occur in this organ following TCDD exposure (18,19).

Limited and conflicting data are available on the effects of TCDD intoxication on the pituitary-adrenal axis. Serum corticosterone concentrations in rats exposed to TCDD have been reported to be both depressed (3,6,14,21) or elevated (6, 10). Depressed serum corticosterone concentrations have been reported at days 4, 7, 13, 14, and 21 following a single, oral dose of 50 μg/kg (3,21) or 100 μg/kg (6,14). This decrease has been proposed to be the result of altered circadian corticosterone secretion (14). However, insufficient time points were presented to determine the actual effect on circadian secretion of corticosterone. Other studies have reported elevated corticosterone concentrations in TCDD-treated rats (6,10). The elevation in corticosterone due to TCDD treatment may be related to the light cycle because the samples were obtained from rats during the late afternoon (6) or from rats subjected to a reverse light cycle (10). Although these reports differ in the nature and timing of the changes, they all suggest that TCDD alters serum corticosterone concentrations.

Synthesis and secretion of corticosterone is dependent upon the anterior pituitary hormone adrenocorticotropin (ACTH). In the adrenal cortex, corticosterone is synthesized

[1] Requests for reprints should be addressed to Walter N. Piper at his current address: Toxicology Program, University of Michigan, M6108 SPH-11, 1420 Washington Heights, Ann Arbor, MI 48109-2129.

from cholesterol with the participation of several monooxygenase reactions catalyzed by different species of mitochondrial and microsomal cytochrome P-450-dependent enzymes. The rate limiting and first step is the conversion of cholesterol to pregnenolone (29), which is catalyzed by mitochondrial P-450_{cscc}. Pregnenolone, the steroid produced, is converted to progesterone. Progesterone then undergoes hydroxylation at the C21 position by microsomal cytochrome P-450 21-hydroxylase (P-450_{C21}) to produce deoxycorticosterone (DOC). In the final step, DOC is converted to corticosterone by mitochondrial cytochrome P-450 11-β hydroxylase (P-$450_{11}\beta$). The conversion of cholesterol to corticosterone, the major glucocorticoid in the rat is dependent upon the trophic effect of ACTH (29,31). ACTH regulates the synthesis of the cytochrome P-450 enzymes in the adrenal cortex by increasing the levels of translatable mRNA that encodes these enzymes (29). Thus, ACTH is necessary in maintaining optimal levels of these steroidogenic enzymes.

Because the synthesis and secretion of corticosterone is dependent upon ACTH, alteration of ACTH may compromise corticosterone biosynthesis. There are a number of potential reactions of corticosteroid biosynthesis that are regulated by ACTH (P-450_{cscc}, P-450_{C21}, P-$450_{11}\beta$) that could be disrupted if ACTH synthesis/secretion was altered by TCDD intoxication. It has been shown that microsomal P-450_{C21} activity is decreased 7 days after TCDD exposure (50 μg/kg; single, oral dose) (20). Because P-450_{C21} is known to be regulated by ACTH, the decreased P-450_{C21} activity may be a result of decreased ACTH concentrations.

Because the anterior pituitary hormone ACTH regulates corticosterone synthesis and secretion, it is possible that the depressed rat adrenal steroidogenesis reported after TCDD treatment may be from decreased anterior pituitary secretion of ACTH. Thus, it would be important to determine whether TCDD could alter circulating plasma ACTH concentrations. This study was designed to assess the ability of TCDD to affect: a) circulating plasma ACTH concentrations, b) circulating plasma corticosterone concentrations, and c) activity of the rate-limiting enzyme, mitochondrial P-450_{cscc}. A depression in these parameters could account for the decreased blood concentrations of corticosterone and the proposed decrease of corticosterone biosynthesis that has been suggested to occur after TCDD treatment. It is possible that any depression of corticosterone biosynthesis caused by TCDD exposure could be due to a decreased adrenal responsiveness to ACTH. Therefore, a second study was conducted to assess the ability of adrenal glands exised from rats treated with TCDD in vivo to respond to a maximally stimulating dose of ACTH in vitro.

METHOD

Animals

TCDD (50 μg/kg) was administered in a single, oral dose to adult, male Sprague-Dawley rats (200–220 g). Sprague-Dawley rats were chosen for their suseptibility to TCDD (3 week LD_{50} of 60 μg/kg). All animals remained alive for the duration of the exposure period and exhibited TCDD-associated weight loss. Acetone-corn oil (1 : 2; 3.8 ml/kg) served as the control vehicle. Rats were handled daily for 7 days prior to TCDD treatment to acclimate the animals to human contact. The animals were housed singly, permitted food and water ad lib, and maintained on a controlled lighting cycle (0600 lights on; 1800 lights off). All experiments were conducted in a controlled, restricted access environment to minimize environmental disturbances for the duration of the experiment. Rats were killed (0900 h) by decapitation, blood was collected in chilled glass tubes containing dry EDTA, and centrifuged. Plasma was separated and frozen at −70°C until hormone assay. The adrenal glands were removed, trimmed of adhering fat, and weighed. Adrenal glands from two animals were pooled and homogenized in 1 ml of 0.1 M sodium phosphate (pH 7.4) containing 0.25 M sucrose using a motor-driven glass Potter-Elvehjem homogenizer and a Teflon pestle (0.15 mm clearance). The homogenate was centrifuged at 600 × g for 10 min. The supernatant was centrifuged at 10,000 × g for 15 min, and the resulting mitochondrial pellet was resuspended in 2 ml of homogenization buffer and recentrifuged at 10,000 × g for 15 min. The pellet was resuspended in 0.5 ml of homogenization buffer and protein content was measured (4).

Measurement of P-450 Cholesterol Side Chain Cleavage Activity

The activity of rat adrenal mitochondrial P-450_{cscc} was determined by measuring the conversion of [1,2,6,7-^{3}H] cholesterol to pregnenolone (12). The assay system contained (final concentrations) 30 μM cholesterol (2 μCi) of [1, 2, 6, 7-^{3}H] cholesterol) and 2.5 mM NADPH in a final volume of 400 μl. The reaction was initiated by the addition of adrenal mitochondrial protein (250 μg). The reaction mixture was incubated at 37°C for 25 min in a shaking water bath. The reaction was terminated by the addition of 0.6 ml of methanol at 5°C. An aliquot (25 μl) of the resulting mixture was subjected to thin-layer chromatography (TLC) (Silica Gel 60, Merck, 20 × 20 cm, 25 mm, without fluorescent indicator) using ethyl

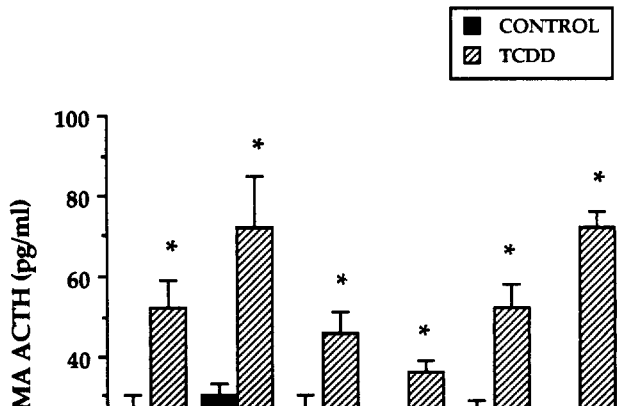

FIG. 1. Effect of TCDD on plasma ACTH concentrations. TCDD (50 μg/kg) was administered in a single, oral dose at day 0. Rats were killed at 0900 h at days 1, 3, 5, 7, 10, and 14 following TCDD treatment. Plasma ACTH concentrations were determined by RIA. Values represent the mean ± SEM for 14 animals at days 1, 3, 5, 10, and 14. Day 7 values represent the mean ± SEM for 24 animals. An asterisk denotes that the difference between TCDD-treated and control values was statistically significant ($p < 0.05$).

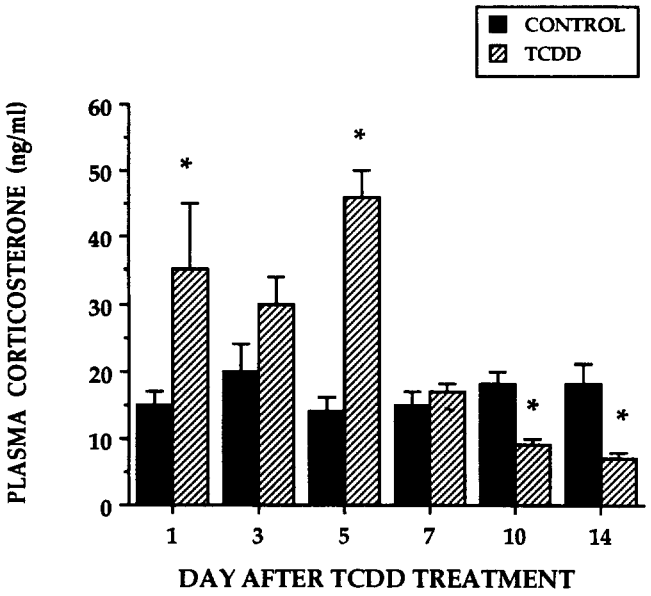

FIG. 2. Effect of TCDD on plasma corticosterone concentrations. TCDD (50 μg/kg) was administered in a single, oral dose at day 0. Rats were killed at 0900 h at days 1, 3, 5, 7, 10, and 14 following TCDD treatment. Plasma corticosterone concentrations were determined by RIA. Values represent the mean ± SEM for 14 animals at days 1, 3, 5, 10, and 14. Day 7 values represent the mean ± SEM for 24 animals. An asterisk denotes that the difference between TCDD-treated and control values was statistically significant ($p < 0.05$).

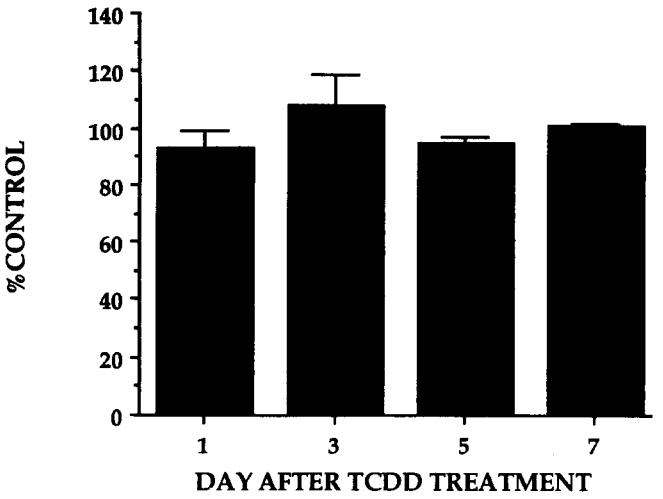

FIG. 3. Effect of TCDD on adrenal mitochondrial P-450$_{cscc}$ activity. TCDD (50 μg/kg) was administered in a single, oral dose at day 0. Rats were killed at 0900 h on days 1, 3, 5, and 7 following TCDD treatment. P-450$_{cscc}$ activity was measured as described in the Method section. Values represent the mean ± SEM for three determinations. Mean control values were 92 ± 26 pmol pregnenolone/mg/min, 67 ± 14, 71 ± 11 and 64 ± 3 for days 1, 3, 5, and 7, respectively.

acetate : toluene (3 : 2, v/v) as the solvent system. A pregnenolone standard was chromatographed along with the unknowns and the area representing [^{3}H]pregnenolone was scraped from the TLC plate and added to 15 ml of Scintiverse BD scintillation fluid (Fisher Scientific; Springfield, NJ). The radioactivity was determined by counting each sample in a Beckman LS8000 liquid scintillation counter. Approximately 90% of the applied radioactivity of standard [^{3}H]pregnenolone was recovered from the TLC plate.

Measurement of Plasma ACTH and Corticosterone Concentrations

Corticosterone was purified to avoid comeasurement of any closely related steroid derivatives (3). An aliquot of plasma (1 ml) was extracted using Bond Elut C-18 columns (Analytichem International; Harbor City, CA). Corticosterone was eluted from the columns with 1 ml of dichloromethane and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in dichloromethane (80 μl) and corticosterone was purified from each sample using TLC (Silica Gel 60, Merck, 20 × 20 cm, 25 mm, without fluorescent indicator; benzene : acetone, 75 : 25, v/v). A corticosterone standard was chromatographed along the unknowns. Using UV light, the bands corresponding to plasma corticosterone samples were scraped from the plate, extracted with 1 ml absolute ethanol, and dried under nitrogen gas. The residue was reconstituted in 0 .1 M sodium phosphate buffered saline, pH 7.4, and stored at −70°C until assayed.

ACTH and corticosterone concentrations were quantitated utilizing commercial radioimmunoassay (RIA) kits (Diagnostic Products Corporation, Los Angeles, CA, and ICN Biochemicals Inc., Costa Mesa, CA, respectively). Intra- and interassay coefficients of variation for ACTH were 6% and 9%, respectively. The sensitivity of the assay was 10 pg/ml. Intra- and interassay coefficients of variation for corticosterone were 7% and 8%, respectively. The sensitivity of the assay was 8 ng/ml.

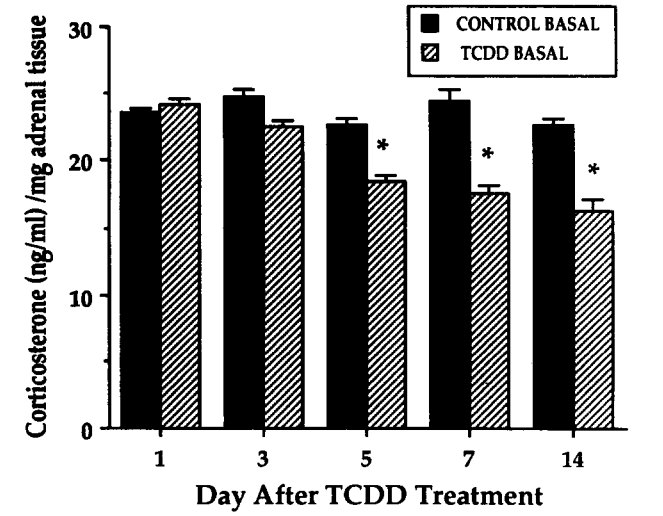

FIG. 4. Basal corticosterone concentrations for control and TCDD-treated quartered adrenals. TCDD (50 μg/kg) was administered in a single, oral dose at day 0. Rats were killed at 0900 h at days 1, 3, 5, 7, and 14. Corticosterone concentrations were measured by RIA as described in the Method section. Values represent the mean ± SEM for 11 determinations. An asterisk denotes that the difference between TCDD-treated and control values was statistically significant ($p < 0.05$).

Measurement of Corticosterone Formation by Quartered Adrenal Glands

Adrenal glands were removed from control or TCDD-treated rats, trimmed of adhering fat, and weighed. Single glands were placed into a petri dish containing medium 199 (M199, pH 7.4) (Gibco BRL; Grand Island, NY) and quartered (28). Quartered adrenal glands were placed into 10 ml Erlenmeyer flasks containing 1.5 ml of M199 and were incubated for 60 min in a shaking water bath at 37°C that was flushed with a slow stream of 5% CO_2 : 95% O_2. At the end of the incubation period, 1.5 ml medium samples were removed and corticosterone content in the medium was quantified by RIA. This was considered basal corticosterone secretion by quartered adrenal glands. Fresh M199 (1.4 ml) containing 100 μl ACTH (100 nM) was then added to each incubation flask. The quartered adrenal glands were stimulated with ACTH (100 nM) for 60 min. Medium samples from the ACTH stimulated adrenal gland (1.5 ml) were removed and corticosterone content was quantified by RIA. Results represent the mean of the values from the adrenal glands of each rat. Corticosterone content in the basal and ACTH stimulated medium samples were measured by utilizing a commercial RIA kit (ICN Biochemicals Inc., Costa Mesa, CA). Sensitivity of the corticosterone RIA was 8 ng/ml plasma and the intra- and interassay coefficients of variation were 7 and 8%, respectively.

Statistical Analysis

Serum ACTH and corticosterone concentrations were analyzed by analysis of variance to test the effects of TCDD, the day following TCDD exposure (day) and the interaction between TCDD and day. The level of significance was set at $p < 0.05$. The statistical differences between means for activity of mitochondrial P-450$_{CSCC}$ were analyzed by Student's t test ($p < 0.05$). Significance of differences between mean values for corticosterone formation by quartered adrenal glands were evaluated by one-way analysis of variance ($p < 0.05$).

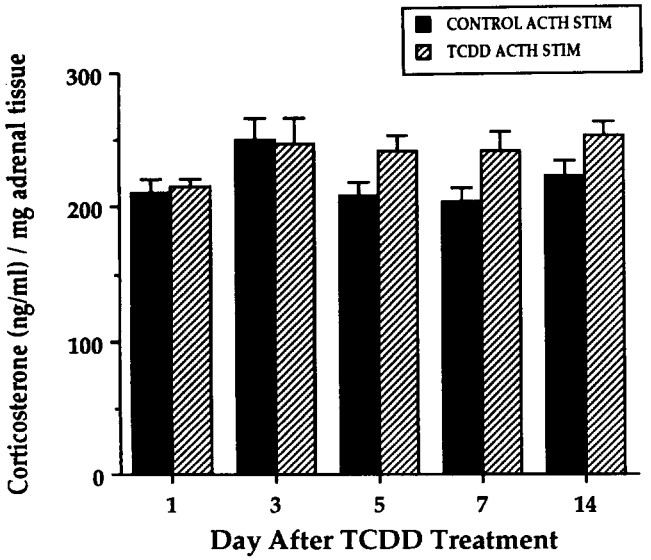

FIG. 5. Corticosterone concentrations for control and TCDD-treated quartered adrenals stimulated with ACTH. TCDD (50 μg/kg) was administered in a single, oral dose at day 0. Rats were killed at 0900 h at days 1, 3, 5, 7, and 14. Corticosterone concentrations were measured by RIA as described in the Method section. Values represent the mean ± SEM for 11 determinations.

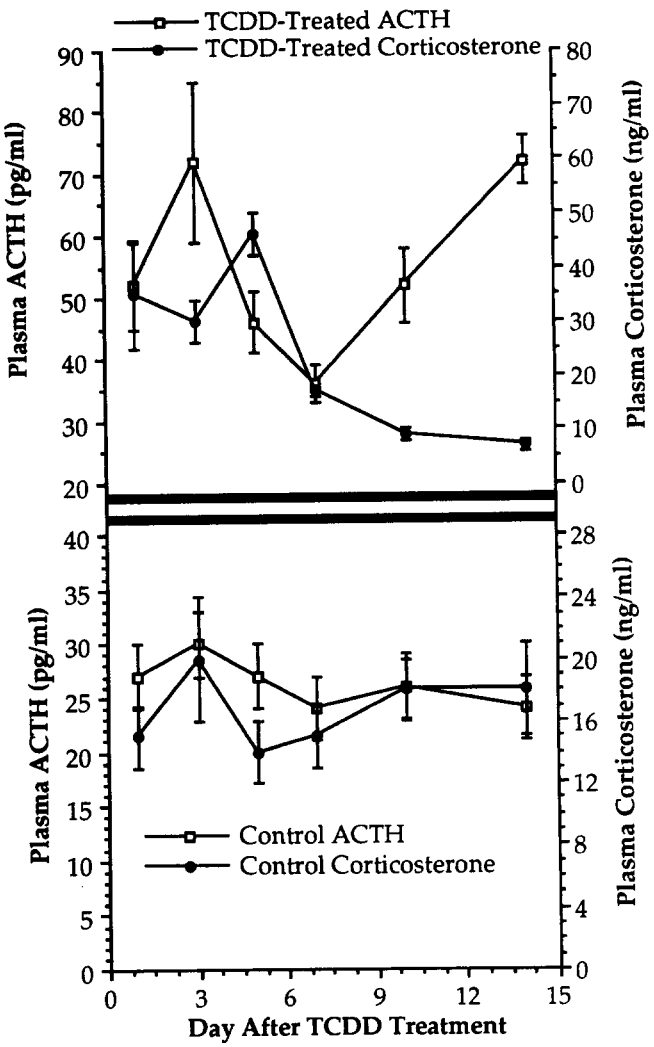

FIG. 6. Secretion pattern of ACTH and corticosterone for control and TCDD-treated rats for days 1 through day 14. TCDD (50 μg/kg) was administered in a single, oral dose at day 0. Rats were killed at 0900 h at days 1, 3, 5, 7, 10, and 14 following TCDD treatment. Plasma ACTH and corticosterone concentrations were determined by RIA. Values represent the mean ± SEM for 14 animals at days 1, 3, 5, 10, and 14. Day 7 values represent the mean ± SEM for 24 animals.

RESULTS

Rat Plasma ACTH Concentrations

Plasma ACTH concentrations were affected by TCDD treatment, day and the TCDD by day interaction ($p < 0.05$). As shown in Fig. 1, exposure to a single oral dose of TCDD resulted in elevated concentrations of plasma ACTH compared to control rats at days 1, 3, 5, 7, 10, and 14 following treatment. Plasma ACTH concentrations were 2.1-, 2.9-, 1.7-, 1.5-, 2.0-, and 3.0-fold greater than controls for these days, respectively.

Rat Plasma Corticosterone Concentrations

Plasma corticosterone concentrations were affected by both TCDD and the TCDD by day interaction ($p < 0.05$, Fig. 2). Plasma corticosterone concentrations were elevated at 1 and 5 days and were depressed at 10 and 14 days following TCDD exposure. At days 1 and 5 plasma corticosterone concentrations in TCDD-treated rats were 5.1- and 8.0-fold greater than control values, respectively. At days 10 and 14 plasma corticosterone concentrations in TCDD-treated rats were depressed to values of 50% and 39% of controls, respectively. At days 2, 3, and 7 plasma corticosterone concentrations were similar in treated and control rats. Plasma corticosterone concentrations were not affected by day ($p > 0.05$).

Activity of Rat Adrenal Mitochondrial Cytochrome P-450$_{CSCC}$

TCDD treatment did not change the activity of the rate-limiting enzyme mitochondrial cytochrome P-450$_{CSCC}$ at days 1 to 7 ($p > 0.05$, Fig. 3).

Corticosterone Secretion by Quartered Adrenal Glands

As shown in Fig. 4, basal medium corticosterone secretion was significantly decreased in adrenal glands from TCDD-treated rats at days 5, 7, and 14. Basal corticosterone concentrations from TCDD-treated rat adrenals were depressed to 81%, 72%, and 71% of control values at days 5, 7, and 14, respectively. Corticosterone secretion by quartered adrenal glands from TCDD-treated rats stimulated with ACTH was equivalent to controls (Fig. 5).

DISCUSSION

Administration of TCDD to adult male rats increased plasma ACTH concentrations as early as 1 day and continued to elevate plasma ACTH concentrations through day 14. This result was not expected based on prior laboratory findings of depressed adrenal microsomal cytochrome P-450 content, decreased microsomal 21-hydroxylase activity, and decreased plasma corticosterone concentrations (20,21). It was hypothesized that TCDD would decrease ACTH concentrations, and that this decrease might, in part, be responsible for the depressed adrenal microsomal cytochrome P-450, decreased microsomal 21-hydroxylase activity, and decreased plasma corticosterone concentrations. In this study, plasma ACTH concentrations were found to be elevated at the same time corticosterone concentrations were found to be depressed (Fig. 6). The secretory pattern of plasma ACTH and corticosterone (CS) by control rats for days 1 through 14 is shown in Fig. 6. Corticosterone secretion appears to be closely regulated by ACTH, with plasma corticosterone levels paralleling those of ACTH. However, Fig. 6 shows a very different pattern for the TCDD-treated rats. Plasma corticosterone concentrations no longer parallel those of ACTH. Quite noticeably, at days 3, 5, 10, and 14, ACTH and corticosterone concentrations appear to be out of phase with each other. ACTH and corticosterone concentrations appear to be totally uncoupled from each other at days 10 and 14. It is possible that TCDD exerts a minor direct effect on the adrenal gland. However, a direct effect of TCDD on the adrenal gland does not appear to be the primary cause of decreased serum corticosterone for the reasons discussed below.

It appears that ACTH is inappropriately regulating corticosterone secretion in rats treated with TCDD. Under normal regulation, elevated plasma ACTH concentrations would result in concordantly elevated plasma corticosterone concentrations. Activity of the rate-limiting enzyme for adrenal steroidogenesis, mitochondrial cytochrome P-450 cholesterol side-chain cleavage (CSCC), was not altered by TCDD-treatment. It has been reported that TCDD-treatment (100 μg/kg; single, oral dose) did not change the activity of rat cytochrome P-450$_{CSCC}$ at day 13 after treatment (6). Cytochrome P-450$_{CSCC}$ activity is normally induced by ACTH. However, the increased plasma ACTH produced by TCDD failed to increase adrenal mitochondrial cytochrome P-450$_{CSCC}$ activity.

These observations (the lack of a consistent increase in plasma corticosterone in response to elevated plasma ACTH and the lack of induction of cytochrome P-450$_{CSCC}$ in response to elevated plasma ACTH) suggest that the adrenal gland may have lost its capacity to respond to ACTH stimulation after TCDD treatment. The second study explored this possibility. Basal corticosterone concentrations secreted into the medium by quartered adrenal glands from TCDD-treated rats were significantly decreased at days 5, 7, and 14, indicating that TCDD-exposure decreases the capacity of the adrenal gland to secrete and/or synthesize corticosterone. However, the ability of quartered adrenal glands from TCDD-treated rats to respond to ACTH stimulation was not altered at any day when compared to controls. These findings indicate that as long as appropriate ACTH is available, TCDD does not seem to interfere with the capacity of the adrenal gland to secrete corticosterone. Thus, it appears as if the decrease in adrenal steroidogenesis that has been suggested to occur after TCDD treatment is not solely because of a decrease in adrenal responsiveness to ACTH. The results reported herein suggest that TCDD may be able to interfere with secretion or synthesis of appropriate, bioactive ACTH from the anterior pituitary gland, which could ultimately compromise adrenal steroidogenesis. Therefore, further experiments are necessary to clarify whether the ACTH secreted by the anterior pituitary has been altered by TCDD treatment.

ACKNOWLEDGEMENTS

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